COMPOUNDS FOR IMMUNODIAGNOSIS OF PROSTATE CANCER AND METHODS FOR THEIR USE

REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of U.S. Patent Application No. 09/020,749, filed February 9, 1998 (Attorney Docket No. 210121.428C2), which is a continuation-in-part of U.S. Patent Application No. 08/904,809, filed August 1, 1997, which is a continuation-in-part of U.S. Patent Application No. 08/806,596, filed February 25, 1997, now abandoned.

TECHNICAL FIELD

The present invention relates generally to the treatment and monitoring of prostate cancer. The invention is more particularly related to polypeptides comprising at least a portion of a prostate protein. Such polypeptides may be used for the production of compounds, such as antibodies, useful for diagnosing and monitoring the progression of prostate cancer, and possibly other tumor types, in a patient.

BACKGROUND OF THE INVENTION

Prostate cancer is the most common form of cancer among males, with an estimated incidence of 30% in men over the age of 50. Overwhelming clinical evidence shows that human prostate cancer has the propensity to metastasize to bone, and the disease appears to progress inevitably from androgen dependent to androgen refractory status, leading to increased patient mortality. This prevalent disease is currently the second leading cause of cancer death among men in the U.S.

In spite of considerable research into diagnosis and therapy of the disease, prostate cancer remains difficult to detect and to treat. Commonly, treatment is based on surgery and/or radiation therapy, but these methods are ineffective in a significant percentage of cases. Two previously identified prostate specific proteins - prostate specific antigen (PSA) and prostatic acid phosphatase (PAP) - have limited diagnostic and therapeutic potential. For example, PSA levels do not always correlate well with the presence of prostate cancer, being positive in a percentage of non-prostate cancer cases, including benign prostatic hyperplasia (BPH). Furthermore, PSA

measurements correlate with prostate volume, and do not indicate the level of metastasis.

Accordingly, there remains a need in the art for improved and diagnostic methods for prostate cancer.

SUMMARY OF THE INVENTION

The present invention provides methods for immunodiagnosis of prostate cancer, together with kits for use in such methods. Polypeptides are disclosed which comprise at least an immunogenic portion of a prostate tumor protein or a variant of said protein that differs only in conservative substitutions and/or modifications, wherein the prostate tumor protein comprises an amino acid sequence encoded by a DNA molecule having a sequence selected from the group consisting of nucleotide sequences recited in SEQ ID Nos: 2-3, 5-107, 109-11, 115-171, 173-175, 177, 179-224 and variants thereof. Such polypeptides may be usefully employed in the diagnosis and monitoring of prostate cancer.

In one specific aspect of the present invention, methods are provided for detecting prostate cancer in a patient, comprising: (a) contacting a biological sample obtained from a patient with a binding agent that is capable of binding to one of the above polypeptides; and (b) detecting in the sample a protein or polypeptide that binds to the binding agent. In preferred embodiments, the binding agent is an antibody, most preferably a monoclonal antibody.

In related aspects, methods are provided for monitoring the progression of prostate cancer in a patient, comprising: (a) contacting a biological sample obtained from a patient with a binding agent that is capable of binding to one of the above polypeptides; (b) determining in the sample an amount of a protein or polypeptide that binds to the binding agent; (c) repeating steps (a) and (b); and comparing the amounts of polypeptide detected in steps (b) and (c).

Within related aspects, the present invention provides antibodies, preferably monoclonal antibodies, that bind to the inventive polypeptides, as well as diagnostic kits comprising such antibodies, and methods of using such antibodies to inhibit the development of prostate cancer.

The present invention further provides methods for detecting prostate cancer comprising: (a) obtaining a biological sample from a patient; (b) contacting the sample with a first and a second oligonucleotide primer in a polymerase chain reaction, at least one of the oligonucleotide primers being specific for a DNA molecule that encodes one of the above polypeptides; and (c) detecting in the sample a DNA sequence that amplifies in the presence of the first and second oligonucleotide primers. In a preferred embodiment, at least one of the oligonucleotide primers comprises at least about 10 contiguous nucleotides of a DNA molecule having a partial sequence selected from the group consisting of SEQ ID Nos: 2-3, 5-107, 109-11, 115-171, 173-175, 177 and 179-224.

In a further aspect, the present invention provides a method for detecting prostate cancer in a patient comprising: (a) obtaining a biological sample from the patient; (b) contacting the sample with an oligonucleotide probe specific for a DNA molecule that encodes one of the above polypeptides; and (c) detecting in the sample a DNA sequence that hybridizes to the oligonucleotide probe. Preferably, the oligonucleotide probe comprises at least about 15 contiguous nucleotides of a DNA molecule having a partial sequence selected from the group consisting of SEQ ID Nos: : 2-3, 5-107, 109-11, 115-171, 173-175, 177 and 179-224.

In related aspects, diagnostic kits comprising the above oligonucleotide probes or primers are provided.

These and other aspects of the present invention will become apparent upon reference to the following detailed description ~~and attached drawings~~. All references disclosed herein are hereby incorporated by reference in their entirety as if each was incorporated individually.

DETAILED DESCRIPTION OF THE INVENTION

As noted above, the present invention is generally directed to compositions and methods for the immunodiagnosis and monitoring of prostate cancer. The inventive compositions are generally polypeptides that comprise at least a portion of a prostate tumor protein. Also included within the present invention are molecules (such as an antibody or fragment thereof) that bind to the inventive polypeptides. Such molecules are referred to herein as "binding agents."

In particular, the subject invention discloses polypeptides comprising at least a portion of a human prostate tumor protein, or a variant thereof such a protein, wherein the prostate tumor protein includes an amino acid sequence encoded by a DNA molecule having a sequence selected from the group consisting of nucleotide sequences recited in SEQ ID Nos: 2-3, 5-107, 109-11, 115-171, 173-175, 177, 179-224, the complements of said nucleotide sequences and variants thereof. As used herein, the term "polypeptide" encompasses amino acid chains of any length, including full length proteins, wherein the amino acid residues are linked by covalent peptide bonds. Thus, a polypeptide comprising a portion of one of the above prostate proteins may consist entirely of the portion, or the portion may be present within a larger polypeptide that contains additional sequences. The additional sequences may be derived from the native protein or may be heterologous, and such sequences may be immunoreactive and/or antigenic.

As used herein, an "immunogenic portion" of a human prostate tumor protein is a portion that is capable of eliciting an immune response in a patient inflicted with prostate cancer and as such binds to antibodies present within sera from a prostate cancer patient. Immunogenic portions of the proteins described herein may thus be identified in antibody binding assays. Such assays may generally be performed using any of a variety of means known to those of ordinary skill in the art, as described, for example, in Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988. For example, a polypeptide may be immobilized on a solid support (as described below) and contacted with patient sera to allow binding of antibodies within the sera to the immobilized polypeptide. Unbound sera may then be removed and bound antibodies detected using, for example, ^{125}I-labeled Protein A. Alternatively, a polypeptide may be used to generate monoclonal and polyclonal antibodies for use in detection of the polypeptide in blood or other fluids of prostate cancer patients.

The compositions and methods of the present invention also encompass variants of the above polypeptides and DNA molecules. A polypeptide "variant," as used herein, is a polypeptide that differs from the recited polypeptide only in conservative substitutions and/or modifications, such that the therapeutic, antigenic and/or immunogenic properties of the polypeptide are retained. Polypeptide variants

preferably exhibit at least about 70%, more preferably at least about 90% and most preferably at least about 95% identity to the identified polypeptides. For prostate tumor polypeptides with immunoreactive properties, variants may, alternatively, be identified by modifying the amino acid sequence of one of the above polypeptides, and evaluating the immunoreactivity of the modified polypeptide. For prostate tumor polypeptides useful for the generation of diagnostic binding agents, a variant may be identified by evaluating a modified polypeptide for the ability to generate antibodies that detect the presence or absence of prostate cancer. Such modified sequences may be prepared and tested using, for example, the representative procedures described herein.

As used herein, a "conservative substitution" is one in which an amino acid is substituted for another amino acid that has similar properties, such that one skilled in the art of peptide chemistry would expect the secondary structure and hydropathic nature of the polypeptide to be substantially unchanged. In general, the following groups of amino acids represent conservative changes: (1) ala, pro, gly, glu, asp, gln, asn, ser, thr; (2) cys, ser, tyr, thr; (3) val, ile, leu, met, ala, phe; (4) lys, arg, his; and (5) phe, tyr, trp, his.

Variants may also, or alternatively, contain other modifications, including the deletion or addition of amino acids that have minimal influence on the antigenic properties, secondary structure and hydropathic nature of the polypeptide. For example, a polypeptide may be conjugated to a signal (or leader) sequence at the N-terminal end of the protein which co-translationally or post-translationally directs transfer of the protein. The polypeptide may also be conjugated to a linker or other sequence for ease of synthesis, purification or identification of the polypeptide (*e.g.*, poly-His), or to enhance binding of the polypeptide to a solid support. For example, a polypeptide may be conjugated to an immunoglobulin Fc region.

A nucleotide "variant" is a sequence that differs from the recited nucleotide sequence in having one or more nucleotide deletions, substitutions or additions. Such modifications may be readily introduced using standard mutagenesis techniques, such as oligonucleotide-directed site-specific mutagenesis as taught, for example, by Adelman et al. (*DNA, 2*:183, 1983). Nucleotide variants may be naturally occurring allelic variants, or non-naturally occurring variants. Variant nucleotide sequences preferably exhibit at least about 70%, more preferably at least about 80% and

most preferably at least about 90% identity to the recited sequence. Such variant nucleotide sequences will generally hybridize to the recite nucleotide sequence under stringent conditions. As used herein, "stringent conditions" refers to prewashing in a solution of 6X SSC, 0.2% SDS; hybridizing at 65 °C, 6X SSC, 0.2% SDS overnight; followed by two washes of 30 minutes each in 1X SSC, 0.1% SDS at 65 °C and two washes of 30 minutes each in 0.2X SSC, 0.1% SDS at 65 °C.

"Polypeptides" as used herein also include combination, or fusion, polypeptides. A "combination polypeptide" is a polypeptide comprising at least one of the above immunogenic portions and one or more additional immunogenic prostate tumor-specific sequences, which are joined via a peptide linkage into a single amino acid chain. The sequences may be joined directly (*i.e.*, with no intervening amino acids) or may be joined by way of a linked sequence (*e.g.*, Gly-Cys-Gly) that does not significantly diminish the immunogenic properties of the component polypeptides.

The prostate tumor proteins of the present invention, and DNA molecules encoding such proteins, may be isolated from prostate tumor tissue using any of a variety of methods well known in the art. DNA sequences corresponding to a gene (of a portion thereof) encoding one of the inventive prostate tumor proteins may be isolated from a prostate tumor cDNA library using a subtraction technique as described in detail below. Examples of such DNA sequences are provided in SEQ ID Nos: 1-107, 109-111, 115-171, 173-175, 177 and 179-224. Partial DNA sequences thus obtained may be used to design oligonucleotide primers for the amplification of full-length DNA sequences in a polymerase chain reaction (PCR), using techniques well known in the art (see, for example, Mullis et al., *Cold Spring Harbor Symp. Quant. Biol.*, *51*:263, 1987; Erlich ed., *PCR Technology*, Stockton Press, NY, 1989). Once a DNA sequence encoding a polypeptide is obtained, any of the above modifications may be readily introduced using standard mutagenesis techniques, such as oligonucleotide-directed site-specific mutagenesis as taught, for example, by Adelman et al. (*DNA*, *2*:183, 1983).

The prostate tumor polypeptides disclosed herein may also be generated by synthetic or recombinant means. Synthetic polypeptides having fewer than about 100 amino acids, and generally fewer than about 50 amino acids, may be generated using techniques well known to those of ordinary skill in the art. For example, such polypeptides may be synthesized using any of the commercially available solid-phase

techniques, such as the Merrifield solid-phase synthesis method, where amino acids are sequentially added to a growing amino acid chain (see, for example, Merrifield, *J. Am. Chem. Soc. 85*:2149-2146, 1963). Equipment for automated synthesis of polypeptides is commercially available from suppliers such as Perkin Elmer/Applied BioSystems Division (Foster City, CA), and may be operated according to the manufacturer's instructions.

Alternatively, any of the above polypeptides may be produced recombinantly by inserting a DNA sequence that encodes the polypeptide into an expression vector and expressing the protein in an appropriate host. Any of a variety of expression vectors known to those of ordinary skill in the art may be employed to express recombinant polypeptides of this invention. Expression may be achieved in any appropriate host cell that has been transformed or transfected with an expression vector containing a DNA molecule that encodes a recombinant polypeptide. Suitable host cells include prokaryotes, yeast and higher eukaryotic cells. Preferably, the host cells employed are *E. coli*, yeast or a mammalian cell line, such as CHO cells. The DNA sequences expressed in this manner may encode naturally occurring polypeptides, portions of naturally occurring polypeptides, or other variants thereof.

In general, regardless of the method of preparation, the polypeptides disclosed herein are prepared in substantially pure form (*i.e.*, the polypeptides are homogenous as determined by amino acid composition and primary sequence analysis). Preferably, the polypeptides are at least about 90% pure, more preferably at least about 95% pure and most preferably at least about 99% pure. In certain embodiments, described in more detail below, the substantially pure polypeptides are incorporated into pharmaceutical compositions or vaccines for use in one or more of the methods disclosed herein.

In a related aspect, the present invention provides fusion proteins comprising a first and a second inventive polypeptide or, alternatively, a polypeptide of the present invention and a known prostate antigen, together with variants of such fusion proteins. The fusion proteins of the present invention may also include a linker peptide between the first and second polypeptides.

A DNA sequence encoding a fusion protein of the present invention is constructed using known recombinant DNA techniques to assemble separate DNA

sequences encoding the first and second polypeptides into an appropriate expression vector. The 3' end of a DNA sequence encoding the first polypeptide is ligated, with or without a peptide linker, to the 5' end of a DNA sequence encoding the second polypeptide so that the reading frames of the sequences are in phase to permit mRNA translation of the two DNA sequences into a single fusion protein that retains the biological activity of both the first and the second polypeptides.

A peptide linker sequence may be employed to separate the first and the second polypeptides by a distance sufficient to ensure that each polypeptide folds into its secondary and tertiary structures. Such a peptide linker sequence is incorporated into the fusion protein using standard techniques well known in the art. Suitable peptide linker sequences may be chosen based on the following factors: (1) their ability to adopt a flexible extended conformation; (2) their inability to adopt a secondary structure that could interact with functional epitopes on the first and second polypeptides; and (3) the lack of hydrophobic or charged residues that might react with the polypeptide functional epitopes. Preferred peptide linker sequences contain Gly, Asn and Ser residues. Other near neutral amino acids, such as Thr and Ala may also be used in the linker sequence. Amino acid sequences which may be usefully employed as linkers include those disclosed in Maratea et al., *Gene 40*:39-46, 1985; Murphy et al., *Proc. Natl. Acad. Sci. USA 83*:8258-8262, 1986; U.S. Patent No. 4,935,233 and U.S. Patent No. 4,751,180. The linker sequence may be from 1 to about 50 amino acids in length. Peptide sequences are not required when the first and second polypeptides have non-essential N-terminal amino acid regions that can be used to separate the functional domains and prevent steric interference.

The ligated DNA sequences are operably linked to suitable transcriptional or translational regulatory elements. The regulatory elements responsible for expression of DNA are located only 5' to the DNA sequence encoding the first polypeptides. Similarly, stop codons require to end translation and transcription termination signals are only present 3' to the DNA sequence encoding the second polypeptide.

Polypeptides and/or fusion proteins of the present invention may be used to generate binding agents, such as antibodies or fragments thereof, that are capable of detecting metastatic human prostate tumors. Binding agents of the present invention

may generally be prepared using methods known to those of ordinary skill in the art, including the representative procedures described herein. Binding agents are capable of differentiating between patients with and without prostate cancer, using the representative assays described herein. In other words, antibodies or other binding agents raised against a prostate tumor protein, or a suitable portion thereof, will generate a signal indicating the presence of primary or metastatic prostate cancer in at least about 20% of patients afflicted with the disease, and will generate a negative signal indicating the absence of the disease in at least about 90% of individuals without primary or metastatic prostate cancer. Suitable portions of such prostate tumor proteins are portions that are able to generate a binding agent that indicates the presence of primary or metastatic prostate cancer in substantially all (*i.e.*, at least about 80%, and preferably at least about 90%) of the patients for which prostate cancer would be indicated using the full length protein, and that indicate the absence of prostate cancer in substantially all of those samples that would be negative when tested with full length protein. The representative assays described below, such as the two-antibody sandwich assay, may generally be employed for evaluating the ability of a binding agent to detect metastatic human prostate tumors.

The ability of a polypeptide and/or fusion protein prepared as described herein to generate antibodies capable of detecting primary or metastatic human prostate tumors may generally be evaluated by raising one or more antibodies against the polypeptide (using, for example, a representative method described herein) and determining the ability of such antibodies to detect such tumors in patients. This determination may be made by assaying biological samples from patients with and without primary or metastatic prostate cancer for the presence of a polypeptide that binds to the generated antibodies. Such test assays may be performed, for example, using a representative procedure described below. Polypeptides that generate antibodies capable of detecting at least 20% of primary or metastatic prostate tumors by such procedures are considered to be useful in assays for detecting primary or metastatic human prostate tumors. Polypeptide specific antibodies may be used alone or in combination to improve sensitivity.

Polypeptides and/or fusion proteins capable of detecting primary or metastatic human prostate tumors may be used as markers for diagnosing prostate

cancer or for monitoring disease progression in patients. In one embodiment, prostate cancer in a patient may be diagnosed by evaluating a biological sample obtained from the patient for the level of one or more of the above polypeptides, relative to a predetermined cut-off value. As used herein, suitable "biological samples" include blood, sera, urine and/or prostate secretions.

The level of one or more of the above polypeptides may be evaluated using any binding agent specific for the polypeptide(s). A "binding agent," in the context of this invention, is any agent (such as a compound or a cell) that binds to a polypeptide as described above. As used herein, "binding" refers to a noncovalent association between two separate molecules (each of which may be free (*i.e.*, in solution) or present on the surface of a cell or a solid support), such that a "complex" is formed. Such a complex may be free or immobilized (either covalently or noncovalently) on a support material. The ability to bind may generally be evaluated by determining a binding constant for the formation of the complex. The binding constant is the value obtained when the concentration of the complex is divided by the product of the component concentrations. In general, two compounds are said to "bind" in the context of the present invention when the binding constant for complex formation exceeds about 10^3 L/mol. The binding constant may be determined using methods well known to those of ordinary skill in the art.

Any agent that satisfies the above requirements may be a binding agent. For example, a binding agent may be a ribosome with or without a peptide component, an RNA molecule or a peptide. In a preferred embodiment, the binding partner is an antibody, or a fragment thereof. Such antibodies may be polyclonal, or monoclonal. In addition, the antibodies may be single chain, chimeric, CDR-grafted or humanized. Antibodies may be prepared by the methods described herein and by other methods well known to those of skill in the art.

There are a variety of assay formats known to those of ordinary skill in the art for using a binding partner to detect polypeptide markers in a sample. *See, e.g.*, Harlow and Lane, *Antibodies: A Laboratory Manual,* Cold Spring Harbor Laboratory, 1988. In a preferred embodiment, the assay involves the use of binding partner immobilized on a solid support to bind to and remove the polypeptide from the remainder of the sample. The bound polypeptide may then be detected using a second

binding partner that contains a reporter group. Suitable second binding partners include antibodies that bind to the binding partner/polypeptide complex. Alternatively, a competitive assay may be utilized, in which a polypeptide is labeled with a reporter group and allowed to bind to the immobilized binding partner after incubation of the binding partner with the sample. The extent to which components of the sample inhibit the binding of the labeled polypeptide to the binding partner is indicative of the reactivity of the sample with the immobilized binding partner.

The solid support may be any material known to those of ordinary skill in the art to which the antigen may be attached. For example, the solid support may be a test well in a microtiter plate or a nitrocellulose or other suitable membrane. Alternatively, the support may be a bead or disc, such as glass, fiberglass, latex or a plastic material such as polystyrene or polyvinylchloride. The support may also be a magnetic particle or a fiber optic sensor, such as those disclosed, for example, in U.S. Patent No. 5,359,681. The binding agent may be immobilized on the solid support using a variety of techniques known to those of skill in the art, which are amply described in the patent and scientific literature. In the context of the present invention, the term "immobilization" refers to both noncovalent association, such as adsorption, and covalent attachment (which may be a direct linkage between the antigen and functional groups on the support or may be a linkage by way of a cross-linking agent). Immobilization by adsorption to a well in a microtiter plate or to a membrane is preferred. In such cases, adsorption may be achieved by contacting the binding agent, in a suitable buffer, with the solid support for a suitable amount of time. The contact time varies with temperature, but is typically between about 1 hour and about 1 day. In general, contacting a well of a plastic microtiter plate (such as polystyrene or polyvinylchloride) with an amount of binding agent ranging from about 10 ng to about 10 μg, and preferably about 100 ng to about 1 μg, is sufficient to immobilize an adequate amount of binding agent.

Covalent attachment of binding agent to a solid support may generally be achieved by first reacting the support with a bifunctional reagent that will react with both the support and a functional group, such as a hydroxyl or amino group, on the binding agent. For example, the binding agent may be covalently attached to supports having an appropriate polymer coating using benzoquinone or by condensation of an

aldehyde group on the support with an amine and an active hydrogen on the binding partner (*see, e.g.*, Pierce Immunotechnology Catalog and Handbook, 1991, at A12-A13).

In certain embodiments, the assay is a two-antibody sandwich assay. This assay may be performed by first contacting an antibody that has been immobilized on a solid support, commonly the well of a microtiter plate, with the sample, such that polypeptides within the sample are allowed to bind to the immobilized antibody. Unbound sample is then removed from the immobilized polypeptide-antibody complexes and a second antibody (containing a reporter group) capable of binding to a different site on the polypeptide is added. The amount of second antibody that remains bound to the solid support is then determined using a method appropriate for the specific reporter group.

More specifically, once the antibody is immobilized on the support as described above, the remaining protein binding sites on the support are typically blocked. Any suitable blocking agent known to those of ordinary skill in the art, such as bovine serum albumin or Tween 20™ (Sigma Chemical Co., St. Louis, MO). The immobilized antibody is then incubated with the sample, and polypeptide is allowed to bind to the antibody. The sample may be diluted with a suitable diluent, such as phosphate-buffered saline (PBS) prior to incubation. In general, an appropriate contact time (*i.e.*, incubation time) is that period of time that is sufficient to detect the presence of polypeptide within a sample obtained from an individual with prostate cancer. Preferably, the contact time is sufficient to achieve a level of binding that is at least about 95% of that achieved at equilibrium between bound and unbound polypeptide. Those of ordinary skill in the art will recognize that the time necessary to achieve equilibrium may be readily determined by assaying the level of binding that occurs over a period of time. At room temperature, an incubation time of about 30 minutes is generally sufficient.

Unbound sample may then be removed by washing the solid support with an appropriate buffer, such as PBS containing 0.1% Tween 20™. The second antibody, which contains a reporter group, may then be added to the solid support. Preferred reporter groups include enzymes (such as horseradish peroxidase), substrates, cofactors, inhibitors, dyes, radionuclides, luminescent groups, fluorescent groups and

biotin. The conjugation of antibody to reporter group may be achieved using standard methods known to those of ordinary skill in the art.

The second antibody is then incubated with the immobilized antibody-polypeptide complex for an amount of time sufficient to detect the bound polypeptide. An appropriate amount of time may generally be determined by assaying the level of binding that occurs over a period of time. Unbound second antibody is then removed and bound second antibody is detected using the reporter group. The method employed for detecting the reporter group depends upon the nature of the reporter group. For radioactive groups, scintillation counting or autoradiographic methods are generally appropriate. Spectroscopic methods may be used to detect dyes, luminescent groups and fluorescent groups. Biotin may be detected using avidin, coupled to a different reporter group (commonly a radioactive or fluorescent group or an enzyme). Enzyme reporter groups may generally be detected by the addition of substrate (generally for a specific period of time), followed by spectroscopic or other analysis of the reaction products.

To determine the presence or absence of prostate cancer, the signal detected from the reporter group that remains bound to the solid support is generally compared to a signal that corresponds to a predetermined cut-off value. In one preferred embodiment, the cut-off value is the average mean signal obtained when the immobilized antibody is incubated with samples from patients without prostate cancer. In general, a sample generating a signal that is three standard deviations above the predetermined cut-off value is considered positive for prostate cancer. In an alternate preferred embodiment, the cut-off value is determined using a Receiver Operator Curve, according to the method of Sackett et al., *Clinical Epidemiology: A Basic Science for Clinical Medicine*, Little Brown and Co., 1985, p. 106-7. Briefly, in this embodiment, the cut-off value may be determined from a plot of pairs of true positive rates (*i.e.*, sensitivity) and false positive rates (100%-specificity) that correspond to each possible cut-off value for the diagnostic test result. The cut-off value on the plot that is the closest to the upper left-hand corner (*i.e.*, the value that encloses the largest area) is the most accurate cut-off value, and a sample generating a signal that is higher than the cut-off value determined by this method may be considered positive. Alternatively, the cut-off value may be shifted to the left along the plot, to minimize the false positive rate, or

to the right, to minimize the false negative rate. In general, a sample generating a signal that is higher than the cut-off value determined by this method is considered positive for prostate cancer.

In a related embodiment, the assay is performed in a flow-through or strip test format, wherein the antibody is immobilized on a membrane, such as nitrocellulose. In the flow-through test, polypeptides within the sample bind to the immobilized antibody as the sample passes through the membrane. A second, labeled antibody then binds to the antibody-polypeptide complex as a solution containing the second antibody flows through the membrane. The detection of bound second antibody may then be performed as described above. In the strip test format, one end of the membrane to which antibody is bound is immersed in a solution containing the sample. The sample migrates along the membrane through a region containing second antibody and to the area of immobilized antibody. Concentration of second antibody at the area of immobilized antibody indicates the presence of prostate cancer. Typically, the concentration of second antibody at that site generates a pattern, such as a line, that can be read visually. The absence of such a pattern indicates a negative result. In general, the amount of antibody immobilized on the membrane is selected to generate a visually discernible pattern when the biological sample contains a level of polypeptide that would be sufficient to generate a positive signal in the two-antibody sandwich assay, in the format discussed above. Preferably, the amount of antibody immobilized on the membrane ranges from about 25 ng to about 1μg, and more preferably from about 50 ng to about 500 ng. Such tests can typically be performed with a very small amount of biological sample.

Of course, numerous other assay protocols exist that are suitable for use with the antigens or antibodies of the present invention. The above descriptions are intended to be exemplary only.

In another embodiment, the above polypeptides may be used as markers for the progression of prostate cancer. In this embodiment, assays as described above for the diagnosis of prostate cancer may be performed over time, and the change in the level of reactive polypeptide(s) evaluated. For example, the assays may be performed every 24-72 hours for a period of 6 months to 1 year, and thereafter performed as needed. In general, prostate cancer is progressing in those patients in whom the level of

polypeptide detected by the binding agent increases over time. In contrast, prostate cancer is not progressing when the level of reactive polypeptide either remains constant or decreases with time.

Antibodies for use in the above methods may be prepared by any of a variety of techniques known to those of ordinary skill in the art. *See, e.g.*, Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, 1988. In one such technique, an immunogen comprising the antigenic polypeptide is initially injected into any of a wide variety of mammals (*e.g.*, mice, rats, rabbits, sheep and goats). In this step, the polypeptides of this invention may serve as the immunogen without modification. Alternatively, particularly for relatively short polypeptides, a superior immune response may be elicited if the polypeptide is joined to a carrier protein, such as bovine serum albumin or keyhole limpet hemocyanin. The immunogen is injected into the animal host, preferably according to a predetermined schedule incorporating one or more booster immunizations, and the animals are bled periodically. Polyclonal antibodies specific for the polypeptide may then be purified from such antisera by, for example, affinity chromatography using the polypeptide coupled to a suitable solid support.

Monoclonal antibodies specific for the antigenic polypeptide of interest may be prepared, for example, using the technique of Kohler and Milstein, *Eur. J. Immunol. 6*:511-519, 1976, and improvements thereto. Briefly, these methods involve the preparation of immortal cell lines capable of producing antibodies having the desired specificity (*i.e.*, reactivity with the polypeptide of interest). Such cell lines may be produced, for example, from spleen cells obtained from an animal immunized as described above. The spleen cells are then immortalized by, for example, fusion with a myeloma cell fusion partner, preferably one that is syngeneic with the immunized animal. A variety of fusion techniques may be employed. For example, the spleen cells and myeloma cells may be combined with a nonionic detergent for a few minutes and then plated at low density on a selective medium that supports the growth of hybrid cells, but not myeloma cells. A preferred selection technique uses HAT (hypoxanthine, aminopterin, thymidine) selection. After a sufficient time, usually about 1 to 2 weeks, colonies of hybrids are observed. Single colonies are selected and tested for binding

activity against the polypeptide. Hybridomas having high reactivity and specificity are preferred.

Monoclonal antibodies may be isolated from the supernatants of growing hybridoma colonies. In addition, various techniques may be employed to enhance the yield, such as injection of the hybridoma cell line into the peritoneal cavity of a suitable vertebrate host, such as a mouse. Monoclonal antibodies may then be harvested from the ascites fluid or the blood. Contaminants may be removed from the antibodies by conventional techniques, such as chromatography, gel filtration, precipitation, and extraction. The polypeptides of this invention may be used in the purification process in, for example, an affinity chromatography step.

Monoclonal antibodies of the present invention may also be used as therapeutic reagents, to diminish or eliminate prostate tumors. The antibodies may be used on their own (for instance, to inhibit metastases) or coupled to one or more therapeutic agents. Suitable agents in this regard include radionuclides, differentiation inducers, drugs, toxins, and derivatives thereof. Preferred radionuclides include ^{90}Y, ^{123}I, ^{125}I, ^{131}I, ^{186}Re, ^{188}Re, ^{211}At, and ^{212}Bi. Preferred drugs include methotrexate, and pyrimidine and purine analogs. Preferred differentiation inducers include phorbol esters and butyric acid. Preferred toxins include ricin, abrin, diptheria toxin, cholera toxin, gelonin, Pseudomonas exotoxin, Shigella toxin, and pokeweed antiviral protein.

A therapeutic agent may be coupled (*e.g.*, covalently bonded) to a suitable monoclonal antibody either directly or indirectly (*e.g.*, via a linker group). A direct reaction between an agent and an antibody is possible when each possesses a substituent capable of reacting with the other. For example, a nucleophilic group, such as an amino or sulfhydryl group, on one may be capable of reacting with a carbonyl-containing group, such as an anhydride or an acid halide, or with an alkyl group containing a good leaving group (*e.g.*, a halide) on the other.

Alternatively, it may be desirable to couple a therapeutic agent and an antibody via a linker group. A linker group can function as a spacer to distance an antibody from an agent in order to avoid interference with binding capabilities. A linker group can also serve to increase the chemical reactivity of a substituent on an agent or an antibody, and thus increase the coupling efficiency. An increase in

chemical reactivity may also facilitate the use of agents, or functional groups on agents, which otherwise would not be possible.

It will be evident to those skilled in the art that a variety of bifunctional or polyfunctional reagents, both homo- and hetero-functional (such as those described in the catalog of the Pierce Chemical Co., Rockford, IL), may be employed as the linker group. Coupling may be effected, for example, through amino groups, carboxyl groups, sulfhydryl groups or oxidized carbohydrate residues. There are numerous references describing such methodology, *e.g.*, U.S. Patent No. 4,671,958, to Rodwell et al.

Where a therapeutic agent is more potent when free from the antibody portion of the immunoconjugates of the present invention, it may be desirable to use a linker group which is cleavable during or upon internalization into a cell. A number of different cleavable linker groups have been described. The mechanisms for the intracellular release of an agent from these linker groups include cleavage by reduction of a disulfide bond (*e.g.*, U.S. Patent No. 4,489,710, to Spitler), by irradiation of a photolabile bond (*e.g.*, U.S. Patent No. 4,625,014, to Senter et al.), by hydrolysis of derivatized amino acid side chains (*e.g.*, U.S. Patent No. 4,638,045, to Kohn et al.), by serum complement-mediated hydrolysis (*e.g.*, U.S. Patent No. 4,671,958, to Rodwell et al.), and acid-catalyzed hydrolysis (*e.g.*, U.S. Patent No. 4,569,789, to Blattler et al.).

It may be desirable to couple more than one agent to an antibody. In one embodiment, multiple molecules of an agent are coupled to one antibody molecule. In another embodiment, more than one type of agent may be coupled to one antibody. Regardless of the particular embodiment, immunoconjugates with more than one agent may be prepared in a variety of ways. For example, more than one agent may be coupled directly to an antibody molecule, or linkers which provide multiple sites for attachment can be used. Alternatively, a carrier can be used.

A carrier may bear the agents in a variety of ways, including covalent bonding either directly or via a linker group. Suitable carriers include proteins such as albumins (*e.g.*, U.S. Patent No. 4,507,234, to Kato et al.), peptides and polysaccharides such as aminodextran (*e.g.*, U.S. Patent No. 4,699,784, to Shih et al.). A carrier may also bear an agent by noncovalent bonding or by encapsulation, such as within a liposome vesicle (*e.g.*, U.S. Patent Nos. 4,429,008 and 4,873,088). Carriers specific for radionuclide agents include radiohalogenated small molecules and chelating

compounds. For example, U.S. Patent No. 4,735,792 discloses representative radiohalogenated small molecules and their synthesis. A radionuclide chelate may be formed from chelating compounds that include those containing nitrogen and sulfur atoms as the donor atoms for binding the metal, or metal oxide, radionuclide. For example, U.S. Patent No. 4,673,562, to Davison et al. discloses representative chelating compounds and their synthesis.

A variety of routes of administration for the antibodies and immunoconjugates may be used. Typically, administration will be intravenous, intramuscular, subcutaneous or in the bed of a resected tumor. It will be evident that the precise dose of the antibody/immunoconjugate will vary depending upon the antibody used, the antigen density on the tumor, and the rate of clearance of the antibody.

Diagnostic reagents of the present invention may also comprise DNA sequences encoding one or more of the above polypeptides, or one or more portions thereof. For example, at least two oligonucleotide primers may be employed in a polymerase chain reaction (PCR) based assay to amplify prostate tumor-specific cDNA derived from a biological sample, wherein at least one of the oligonucleotide primers is specific for a DNA molecule encoding a prostate tumor protein of the present invention. The presence of the amplified cDNA is then detected using techniques well known in the art, such as gel electrophoresis. Similarly, oligonucleotide probes specific for a DNA molecule encoding a prostate tumor protein of the present invention may be used in a hybridization assay to detect the presence of an inventive polypeptide in a biological sample.

As used herein, the term "oligonucleotide primer/probe specific for a DNA molecule" means an oligonucleotide sequence that has at least about 80%, preferably at least about 90% and more preferably at least about 95%, identity to the DNA molecule in question. Oligonucleotide primers and/or probes which may be usefully employed in the inventive diagnostic methods preferably have at least about 10-40 nucleotides. In a preferred embodiment, the oligonucleotide primers comprise at least about 10 contiguous nucleotides of a DNA molecule having a sequence selected from SEQ ID Nos: 1-107, 109-111, 115-171, 173-175, 177 and 179-224. Preferably, oligonucleotide probes for use in the inventive diagnostic methods comprise at least about 15 contiguous oligonucleotides of a DNA molecule having a sequence provided

in SEQ ID Nos: 1-107, 109-111, 115-171, 173-175, 177 and 179-224. Techniques for both PCR based assays and hybridization assays are well known in the art (see, for example, Mullis et al. *Ibid*; Ehrlich, *Ibid*). Primers or probes may thus be used to detect prostate tumor-specific sequences in biological samples, including blood, semen, prostate tissue and/or prostate tumor tissue.

Polypeptides of the present invention that comprise an immunogenic portion of a prostate tumor protein may also be used for immunotherapy of prostate cancer, wherein the polypeptide stimulates the patient's own immune response to prostate tumor cells. In further aspects, the present invention provides methods for using one or more of the immunoreactive polypeptides encoded by a DNA molecule having a sequence provided in SEQ ID NO: 1-107, 109-111, 115-171, 173-175, 177 and 179-224 (or DNA encoding such polypeptides) for immunotherapy of prostate cancer in a patient. As used herein, a "patient" refers to any warm-blooded animal, preferably a human. A patient may be afflicted with a disease, or may be free of detectable disease. Accordingly, the above immunoreactive polypeptides may be used to treat prostate cancer or to inhibit the development of prostate cancer. The polypeptides may be administered either prior to or following surgical removal of primary tumors and/or treatment by administration of radiotherapy and conventional chemotherapeutic drugs.

In these aspects, the polypeptide is generally present within a pharmaceutical composition and/or a vaccine. Pharmaceutical compositions may comprise one or more polypeptides, each of which may contain one or more of the above sequences (or variants thereof), and a physiologically acceptable carrier. The vaccines may comprise one or more of such polypeptides and a non-specific immune response enhancer, such as an adjuvant, biodegradable microsphere (*e.g.*, polylactic galactide) or a liposome (into which the polypeptide is incorporated). Pharmaceutical compositions and vaccines may also contain other epitopes of prostate tumor antigens, either incorporated into a combination polypeptide (*i.e.*, a single polypeptide that contains multiple epitopes) or present within a separate polypeptide.

Alternatively, a pharmaceutical composition or vaccine may contain DNA encoding one or more of the above polypeptides, such that the polypeptide is generated *in situ*. In such pharmaceutical compositions and vaccines, the DNA may be present within any of a variety of delivery systems known to those of ordinary skill in

the art, including nucleic acid expression systems, bacteria and viral expression systems. Appropriate nucleic acid expression systems contain the necessary DNA sequences for expression in the patient (such as a suitable promoter). Bacterial delivery systems involve the administration of a bacterium (such as *Bacillus-Calmette-Guerrin*) that expresses an epitope of a prostate cell antigen on its cell surface. In a preferred embodiment, the DNA may be introduced using a viral expression system (*e.g.*, vaccinia or other pox virus, retrovirus, or adenovirus), which may involve the use of a non-pathogenic (defective), replication competent virus. Suitable systems are disclosed, for example, in Fisher-Hoch et al., *PNAS 86*:317-321, 1989; Flexner et al., *Ann. N.Y. Acad. Sci. 569*:86-103, 1989; Flexner et al., *Vaccine 8*:17-21, 1990; U.S. Patent Nos. 4,603,112, 4,769,330, and 5,017,487; WO 89/01973; U.S. Patent No. 4,777,127; GB 2,200,651; EP 0,345,242; WO 91/02805; Berkner, *Biotechniques 6*:616-627, 1988; Rosenfeld et al., *Science 252*:431-434, 1991; Kolls et al., *PNAS 91*:215-219, 1994; Kass-Eisler et al., *PNAS 90*:11498-11502, 1993; Guzman et al., *Circulation 88*:2838-2848, 1993; and Guzman et al., *Cir. Res. 73*:1202-1207, 1993. Techniques for incorporating DNA into such expression systems are well known to those of ordinary skill in the art. The DNA may also be "naked," as described, for example, in published PCT application WO 90/11092, and Ulmer et al., *Science 259*:1745-1749, 1993, reviewed by Cohen, *Science 259*:1691-1692, 1993. The uptake of naked DNA may be increased by coating the DNA onto biodegradable beads, which are efficiently transported into the cells.

Routes and frequency of administration, as well as dosage, will vary from individual to individual and may parallel those currently being used in immunotherapy of other diseases. In general, the pharmaceutical compositions and vaccines may be administered by injection (*e.g.*, intracutaneous, intramuscular, intravenous or subcutaneous), intranasally (*e.g.*, by aspiration) or orally. Between 1 and 10 doses may be administered over a 3-24 week period. Preferably, 4 doses are administered, at an interval of 3 months, and booster administrations may be given periodically thereafter. Alternate protocols may be appropriate for individual patients. A suitable dose is an amount of polypeptide or DNA that is effective to raise an immune response (cellular and/or humoral) against prostate tumor cells in a treated patient. A suitable immune response is at least 10-50% above the basal (*i.e.*, untreated) level. In

general, the amount of polypeptide present in a dose (or produced *in situ* by the DNA in a dose) ranges from about 1 pg to about 100 mg per kg of host, typically from about 10 pg to about 1 mg, and preferably from about 100 pg to about 1 μg. Suitable dose sizes will vary with the size of the patient, but will typically range from about 0.01 mL to about 5 mL.

While any suitable carrier known to those of ordinary skill in the art may be employed in the pharmaceutical compositions of this invention, the type of carrier will vary depending on the mode of administration. For parenteral administration, such as subcutaneous injection, the carrier preferably comprises water, saline, alcohol, a lipid, a wax and/or a buffer. For oral administration, any of the above carriers or a solid carrier, such as mannitol, lactose, starch, magnesium stearate, sodium saccharine, talcum, cellulose, glucose, sucrose, and/or magnesium carbonate, may be employed. Biodegradable microspheres (*e.g.*, polylactic glycolide) may also be employed as carriers for the pharmaceutical compositions of this invention. Suitable biodegradable microspheres are disclosed, for example, in U.S. Patent Nos. 4,897,268 and 5,075,109.

Any of a variety of non-specific immune response enhancers may be employed in the vaccines of this invention. For example, an adjuvant may be included. Most adjuvants contain a substance designed to protect the antigen from rapid catabolism, such as aluminum hydroxide or mineral oil, and a nonspecific stimulator of immune response, such as lipid A, *Bordella pertussis* or *Mycobacterium tuberculosis*. Such adjuvants are commercially available as, for example, Freund's Incomplete Adjuvant and Complete Adjuvant (Difco Laboratories, Detroit, MI) and Merck Adjuvant 65 (Merck and Company, Inc., Rahway, NJ).

Polypeptides disclosed herein may also be employed in *ex vivo* treatment of prostate cancer. For example, cells of the immune system, such as T cells, may be isolated from the peripheral blood of a patient, using a commercially available cell separation system, such as CellPro Incorporated's (Bothell, WA) CEPRATE™ system (see U.S. Patent No. 5,240,856; U.S. Patent No. 5,215,926; WO 89/06280; WO 91/16116 and WO 92/07243). The separated cells are stimulated with one or more of the immunoreactive polypeptides contained within a delivery vehicle, such as a microsphere, to provide antigen-specific T cells. The population of tumor antigen-

specific T cells is then expanded using standard techniques and the cells are administered back to the patient.

The following Examples are offered by way of illustration and not by way of limitation.

EXAMPLES

EXAMPLE 1

ISOLATION AND CHARACTERIZATION OF PROSTATE TUMOR POLYPEPTIDES

This Example describes the isolation of prostate tumor polypeptides from a prostate tumor cDNA library.

A human prostate tumor cDNA expression library was constructed from prostate tumor poly A^+ RNA using a Superscript Plasmid System for cDNA Synthesis and Plasmid Cloning kit (BRL Life Technologies, Gaithersburg, MD 20897) following the manufacturer's protocol. Specifically, prostate tumor tissues were homogenized with polytron (Kinematica, Switzerland) and total RNA was extracted using Trizol reagent (BRL Life Technologies) as directed by the manufacturer. The poly A^+ RNA was then purified using a Qiagen oligotex spin column mRNA purification kit (Qiagen, Santa Clarita, CA 91355) according to the manufacturer's protocol. First-strand cDNA was synthesized using the NotI/Oligo-dT18 primer. Double-stranded cDNA was synthesized, ligated with EcoRI/BAXI adaptors (Invitrogen, San Diego, CA) and digested with NotI. Following size fractionation with Chroma Spin-1000 columns (Clontech, Palo Alto, CA 94303), the cDNA was ligated into the EcoRI/NotI site of pCDNA3.1 (Invitrogen) and transformed into ElectroMax *E. coli* DH10B cells (BRL Life Technologies) by electroporation.

Using the same procedure, a normal human pancreas cDNA expression library was prepared from a pool of six tissue specimens (Clontech). The cDNA libraries were characterized by determining the number of independent colonies, the percentage of clones that carried insert, the average insert size and by sequence analysis. The prostate tumor library contained 1.64×10^7 independent colonies, with 70% of

clones having an insert and the average insert size being 1745 base pairs. The normal pancreas cDNA library contained 3.3 x 10^6 independent colonies, with 69% of clones having inserts and the average insert size being 1120 base pairs. For both libraries, sequence analysis showed that the majority of clones had a full length cDNA sequence and were synthesized from mRNA, with minimal rRNA and mitochondrial DNA contamination.

cDNA library subtraction was performed using the above prostate tumor and normal pancreas cDNA libraries, as described by Hara et al. (*Blood*, *84*:189-199, 1994) with some modifications. Specifically, a prostate tumor-specific subtracted cDNA library was generated as follows. Normal pancreas cDNA library (70 μg) was digested with EcoRI, NotI, and SfuI, followed by a filling-in reaction with DNA polymerase Klenow fragment. After phenol-chloroform extraction and ethanol precipitation, the DNA was dissolved in 100 μl of H_2O, heat-denatured and mixed with 100 μl (100 μg) of Photoprobe biotin (Vector Laboratories, Burlingame, CA). As recommended by the manufacturer, the resulting mixture was irradiated with a 270 W sunlamp on ice for 20 minutes. Additional Photoprobe biotin (50 μl) was added and the biotinylation reaction was repeated. After extraction with butanol five times, the DNA was ethanol-precipitated and dissolved in 23 μl H_2O to form the driver DNA.

To form the tracer DNA, 10 μg prostate tumor cDNA library was digested with BamHI and XhoI, phenol chloroform extracted and passed through Chroma spin-400 columns (Clontech). Following ethanol precipitation, the tracer DNA was dissolved in 5 μl H_2O. Tracer DNA was mixed with 15 μl driver DNA and 20 μl of 2 x hybridization buffer (1.5 M NaCl/10 mM EDTA/50 mM HEPES pH 7.5/0.2% sodium dodecyl sulfate), overlaid with mineral oil, and heat-denatured completely. The sample was immediately transferred into a 68 °C water bath and incubated for 20 hours (long hybridization [LH]). The reaction mixture was then subjected to a streptavidin treatment followed by phenol/chloroform extraction. This process was repeated three more times. Subtracted DNA was precipitated, dissolved in 12 μl H_2O, mixed with 8 μl driver DNA and 20 μl of 2 x hybridization buffer, and subjected to a hybridization at 68 °C for 2 hours (short hybridization [SH]). After removal of biotinylated double-stranded DNA, subtracted cDNA was ligated into BamHI/XhoI site of chloramphenicol

resistant pBCSK$^+$ (Stratagene, La Jolla, CA 92037) and transformed into ElectroMax *E. coli* DH10B cells by electroporation to generate a prostate tumor specific subtracted cDNA library(prostate subtraction 1.

To analyze the subtracted cDNA library, plasmid DNA was prepared from 100 independent clones, randomly picked from the subtracted prostate tumor specific library and grouped based on insert size. Representative cDNA clones were further characterized by DNA sequencing with a Perkin Elmer/Applied Biosystems Division Automated Sequencer Model 373A (Foster City, CA). Six cDNA clones, hereinafter referred to as F1-13, F1-12, F1-16, H1-1, H1-9 and H1-4, were shown to be abundant in the subtracted prostate-specific cDNA library. The determined 3' and 5' cDNA sequences for F1-12 are provided in SEQ ID NO: 2 and 3, respectively, with determined 3' cDNA sequences for F1-13, F1-16, H1-1, H1-9 and H1-4 being provided in SEQ ID No: 1 and 4-7, respectively.

The cDNA sequences for the isolated clones were compared to known sequences in the gene bank using the EMBL and GenBank databases (release 96). Four of the prostate tumor cDNA clones, F1-13, F1-16, H1-1, and H1-4, were determined to encode the following previously identified proteins: prostate specific antigen (PSA), human glandular kallikrein, human tumor expression enhanced gene, and mitochondria cytochrome C oxidase subunit II. H1-9 was found to be identical to a previously identified human autonomously replicating sequence. No significant homologies to the cDNA sequence for F1-12 were found.

Subsequent studies led to the isolation of a full-length cDNA sequence for F1-12. This sequence is provided in SEQ ID NO: 107, with the corresponding predicted amino acid sequence being provided in SEQ ID NO: 108.

To clone less abundant prostate tumor specific genes, cDNA library subtraction was performed by subtracting the prostate tumor cDNA library described above with the normal pancreas cDNA library and with the three most abundant genes in the previously subtracted prostate tumor specific cDNA library: human glandular kallikrein, prostate specific antigen (PSA), and mitochondria cytochrome C oxidase subunit II. Specifically, 1 μg each of human glandular kallikrein, PSA and mitochondria cytochrome C oxidase subunit II cDNAs in pCDNA3.1 were added to the driver DNA and subtraction was performed as described above to provide a second

subtracted cDNA library hereinafter referred to as the "subtracted prostate tumor specific cDNA library with spike".

Twenty-two cDNA clones were isolated from the subtracted prostate tumor specific cDNA library with spike. The determined 3' and 5' cDNA sequences for the clones referred to as J1-17, L1-12, N1-1862, J1-13, J1-19, J1-25, J1-24, K1-58, K1-63, L1-4 and L1-14 are provided in SEQ ID Nos: 8-9, 10-11, 12-13, 14-15, 16-17, 18-19, 20-21, 22-23, 24-25, 26-27 and 28-29, respectively. The determined 3' cDNA sequences for the clones referred to as J1-12, J1-16, J1-21, K1-48, K1-55, L1-2, L1-6, N1-1858, N1-1860, N1-1861, N1-1864 are provided in SEQ ID Nos: 30-40, respectively. Comparison of these sequences with those in the gene bank as described above, revealed no significant homologies to three of the five most abundant DNA species, (J1-17, L1-12 and N1-1862; SEQ ID Nos: 8-9, 10-11 and 12-13, respectively). Of the remaining two most abundant species, one (J1-12; SEQ ID NO:30) was found to be identical to the previously identified human pulmonary surfactant-associated protein, and the other (K1-48; SEQ ID NO:33) was determined to have some homology to *R. norvegicus* mRNA for 2-arylpropionyl-CoA epimerase. Of the 17 less abundant cDNA clones isolated from the subtracted prostate tumor specific cDNA library with spike, four (J1-16, K1-55, L1-6 and N1-1864; SEQ ID Nos:31, 34, 36 and 40, respectively) were found to be identical to previously identified sequences, two (J1-21 and N1-1860; SEQ ID Nos: 32 and 38, respectively) were found to show some homology to non-human sequences, and two (L1-2 and N1-1861; SEQ ID Nos: 35 and 39, respectively) were found to show some homology to known human sequences. No significant homologies were found to the polypeptides J1-13, J1-19, J1-24, J1-25, K1-58, K1-63, L1-4, L1-14 (SEQ ID Nos: 14-15, 16-17, 20-21, 18-19, 22-23, 24-25, 26-27, 28-29, respectively).

Subsequent studies led to the isolation of full length cDNA sequences for J1-17, L1-12 and N1-1862 (SEQ ID NOS: 109-111, respectively). The corresponding predicted amino acid sequences are provided in SEQ ID NOS: 112-114.

In a further experiment, four additional clones were identified by subtracting a prostate tumor cDNA library with normal prostate cDNA prepared from a pool of three normal prostate poly A+ RNA (prostate subtraction 2). The determined cDNA sequences for these clones, hereinafter referred to as U1-3064, U1-3065, V1-

3692 and 1A-3905, are provided in SEQ ID NO: 69-72, respectively. Comparison of the determined sequences with those in the gene bank revealed no significant homologies to U1-3065.

A second subtraction with spike (prostate subtraction spike 2) was performed by subtracting a prostate tumor specific cDNA library with spike with normal pancreas cDNA library and further spiked with PSA, J1-17, pulmonary surfactant-associated protein, mitochondrial DNA, cytochrome c oxidase subunit II, N1-1862, autonomously replicating sequence, L1-12 and tumor expression enhanced gene. Four additional clones, hereinafter referred to as V1-3686, R1-2330, 1B-3976 and V1-3679, were isolated. The determined cDNA sequences for these clones are provided in SEQ ID NO:73-76, respectively. Comparison of these sequences with those in the gene bank revealed no significant homologies to V1-3686 and R1-2330.

Further analysis of the three prostate subtractions described above (prostate subtraction 2, subtracted prostate tumor specific cDNA library with spike, and prostate subtraction spike 2) resulted in the identification of sixteen additional clones, referred to as 1G-4736, 1G-4738, 1G-4741, 1G-4744, 1G-4734, 1H-4774, 1H-4781, 1H-4785, 1H-4787, 1H-4796, 1I-4810, 1I-4811, 1J-4876, 1K-4884 and 1K-4896. The determined cDNA sequences for these clones are provided in SEQ ID NOS: 77-92, respectively. Comparison of these sequences with those in the gene bank as described above, revealed no significant homologies to 1G-4741, 1G-4734, 1I-4807, 1J-4876 and 1K-4896 (SEQ ID NOS: 79, 81, 87, 90 and 92, respectively). Further analysis of the isolated clones led to the determination of extended cDNA sequences for 1G-4736, 1G-4738, 1G-4741, 1G-4744, 1H-4774, 1H-4781, 1H-4785, 1H-4787, 1H-4796, 1I-4807, 1J-4876, 1K-4884 and 1K-4896, provided in SEQ ID NOS: 179-188 and 191-193, respectively, and to the determination of additional partial cDNA sequences for 1I-4810 and 1I-4811, provided in SEQ ID NOS: 189 and 190, respectively.

An additional subtraction was performed by subtracting a normal prostate cDNA library with normal pancreas cDNA (prostate subtraction 3). This led to the identification of six additional clones referred to as 1G-4761, 1G-4762, 1H-4766, 1H-4770, 1H-4771 and 1H-4772 (SEQ ID NOS: 93-98). Comparison of these sequences with those in the gene bank revealed no significant homologies to 1G-4761 and 1H-4771 (SEQ ID NOS: 93 and 97, respectively). Further analysis of the isolated

clones led to the determination of extended cDNA sequences for 1G-4761, 1G-4762, 1H-4766 and 1H-4772 provided in SEQ ID NOS: 194-196 and 199, respectively, and to the determination of additional partial cDNA sequences for 1H-4770 and 1H-4771, provided in SEQ ID NOS: 197 and 198, respectively.

Subtraction of a prostate tumor cDNA library, prepared from a pool of polyA+ RNA from three prostate cancer patients, with a normal pancreas cDNA library (prostate subtraction 4) led to the identification of eight clones, referred to as 1D-4297, 1D-4309, 1D.1-4278, 1D-4288, 1D-4283, 1D-4304, 1D-4296 and 1D-4280 (SEQ ID NOS: 99-107). These sequences were compared to those in the gene bank as described above. No significant homologies were found to 1D-4283 and 1D-4304 (SEQ ID NOS: 103 and 104, respectively). Further analysis of the isolated clones led to the determination of extended cDNA sequences for 1D-4309, 1D.1-4278, 1D-4288, 1D-4283, 1D-4304, 1D-4296 and 1D-4280, provided in SEQ ID NOS: 200-206, respectively.

cDNA clones isolated in prostate subtraction 1 and prostate subtraction 2, described above, were colony PCR amplified and their mRNA expression levels in prostate tumor, normal prostate and in various other normal tissues were determined using microarray technology (Synteni, Palo Alto, CA). Briefly, the PCR amplification products were dotted onto slides in an array format, with each product occupying a unique location in the array. mRNA was extracted from the tissue sample to be tested, reverse transcribed, and fluorescent-labeled cDNA probes were generated. The microarrays were probed with the labeled cDNA probes, the slides scanned and fluorescence intensity was measured. This intensity correlates with the hybridization intensity. Two novel clones (referred to as P509S and P510S) were found to be over-expressed in prostate tumor and normal prostate and expressed at low levels in all other normal tissues tested (liver, pancreas, skin, bone marrow, brain, breast, adrenal gland, bladder, testes, salivary gland, large intestine, kidney, ovary, lung, spinal cord, skeletal muscle and colon). The determined cDNA sequences for P509S and P510S are provided in SEQ ID NO: 223 and 224, respectively. Comparison of these sequences with those in the gene bank as described above, revealed some homology to previously identified ESTs.

EXAMPLE 2

DETERMINATION OF TISSUE SPECIFICITY OF PROSTATE TUMOR POLYPEPTIDES

Using gene specific primers, mRNA expression levels for the representative prostate tumor polypeptides F1-16, H1-1, J1-17, L1-12, F1-12 and N1-1862 were examined in a variety of normal and tumor tissues using RT-PCR.

Briefly, total RNA was extracted from a variety of normal and tumor tissues using Trizol reagent as described above. First strand synthesis was carried out using 1-2 μg of total RNA with SuperScript II reverse transcriptase (BRL Life Technologies) at 42 °C for one hour. The cDNA was then amplified by PCR with gene-specific primers. To ensure the semi-quantitative nature of the RT-PCR, β-actin was used as an internal control for each of the tissues examined. First, serial dilutions of the first strand cDNAs were prepared and RT-PCR assays were performed using β-actin specific primers. A dilution was then chosen that enabled the linear range amplification of the β-actin template and which was sensitive enough to reflect the differences in the initial copy numbers. Using these conditions, the β-actin levels were determined for each reverse transcription reaction from each tissue. DNA contamination was minimized by DNase treatment and by assuring a negative PCR result when using first strand cDNA that was prepared without adding reverse transcriptase.

mRNA Expression levels were examined in four different types of tumor tissue (prostate tumor from 2 patients, breast tumor from 3 patients, colon tumor, lung tumor), and sixteen different normal tissues, including prostate, colon, kidney, liver, lung, ovary, pancreas, skeletal muscle, skin, stomach, testes, bone marrow and brain. F1-16 was found to be expressed at high levels in prostate tumor tissue, colon tumor and normal prostate, and at lower levels in normal liver, skin and testes, with expression being undetectable in the other tissues examined. H1-1 was found to be expressed at high levels in prostate tumor, lung tumor, breast tumor, normal prostate, normal colon and normal brain, at much lower levels in normal lung, pancreas, skeletal muscle, skin, small intestine, bone marrow, and was not detected in the other tissues tested. J1-17

and L1-12 appear to be specifically over-expressed in prostate, with both genes being expressed at high levels in prostate tumor and normal prostate but at low to undetectable levels in all the other tissues examined. N1-1862 was found to be over-expressed in 60% of prostate tumors and detectable in normal colon and kidney. The RT-PCR results thus indicate that F1-16, H1-1, J1-17, N1-1862 and L1-12 are either prostate specific or are expressed at significantly elevated levels in prostate.

Further RT-PCR studies showed that F1-12 is over-expressed in 60% of prostate tumors, detectable in normal kidney but not detectable in all other tissues tested. Similarly, R1-2330 was shown to be over-expressed in 40% of prostate tumors, detectable in normal kidney and liver, but not detectable in all other tissues tested. U1-3064 was found to be over-expressed in 60% of prostate tumors, and also expressed in breast and colon tumors, but was not detectable in normal tissues.

RT-PCR characterization of R1-2330, U1-3064 and 1D-4279 showed that these three antigens are over-expressed in prostate and/or prostate tumors.

Northern analysis with four prostate tumors, two normal prostate samples, two BPH prostates, and normal colon, kidney, liver, lung, pancrease, skeletal muscle, brain, stomach, testes, small intestine and bone marrow, showed that L1-12 is over-expressed in prostate tumors and normal prostate, while being undetectable in other normal tissues tested. J1-17 was detected in two prostate tumors and not in the other tissues tested. N1-1862 was found to be over-expressed in three prostate tumors and to be expressed in normal prostate, colon and kidney, but not in other tissues tested. F1-12 was found to be highly expressed in two prostate tumors and to be undetectable in all other tissues tested.

The micro-array technology described above was used to determine the expression levels of representative antigens described herein in prostate tumor, breast tumor and the following normal tissues: prostate, liver, pancreas, skin, bone marrow, brain, breast, adrenal gland, bladder, testes, salivary gland, large intestine, kidney, ovary, lung, spinal cord, skeletal muscle and colon. L1-12 was found to be over-expressed in normal prostate and prostate tumor, with some expression being detected in normal skeletal muscle. Both J1-12 and F1-12 were found to be over-expressed in prostate tumor, with expression being lower or undetectable in all other tissues tested. N1-1862 was found to be expressed at high levels in prostate tumor and normal

prostate, and at low levels in normal large intestine and normal colon, with expression being undetectable in all other tissues tested. R1-2330 was found to be over-expressed in prostate tumor and normal prostate, and to be expressed at lower levels in all other tissues tested. 1D-4279 was found to be over-expressed in prostate tumor and normal prostate, expressed at lower levels in normal spinal cord, and to be undetectable in all other tissues tested.

Example 3

ISOLATION AND CHARACTERIZATION OF PROSTATE TUMOR POLYPEPTIDES BY PCR-BASED SUBTRACTON

A cDNA subtraction library, containing cDNA from normal prostate subtracted with ten other normal tissue cDNAs (brain, heart, kidney, liver, lung, ovary, placenta, skeletal muscle, spleen and thymus) and then submitted to a first round of PCR amplification, was purchased from Clontech. This library was subjected to a second round of PCR amplification, following the manufacturer's protocol. The resulting cDNA fragments were subcloned into the vector pT7 Blue T-vector (Novagen, Madison, WI) and transformed into XL-1 Blue MRF' *E. coli* (Stratagene). DNA was isolated from independent clones and sequenced using a Perkin Elmer/Applied Biosystems Division Automated Sequencer Model 373A.

Fifty-nine positive clones were sequenced. Comparison of the DNA sequences of these clones with those in the gene bank, as described above, revealed no significant homologies to 25 of these clones, hereinafter referred to as P5, P8, P9, P18, P20, P30, P34, P36, P38, P39, P42, P49, P50, P53, P55, P60, P64, P65, P73, P75, P76, P79, and P84. The determined cDNA sequences for these clones are provided in SEQ ID NO:41-45, 47-52 and 54-65, respectively. P29, P47, P68, P80 and P82 (SEQ ID NO:46, 53 and 66-68, respectively) were found to show some degree of homology to previously identified DNA sequences. To the best of the inventors' knowledge, none of these sequences have been previously shown to be present in prostate.

Further studies using the PCR-based methodology described above resulted in the isolation of more than 180 additional clones, of which 23 clones were

found to show no significant homologies to known sequences. The determined cDNA sequences for these clones are provided in SEQ ID NO: 115-123, 127, 131, 137, 145, 147-151, 153, 156-158 and 160. Twenty-three clones (SEQ ID NO: 124-126, 128-130, 132-136, 138-144, 146, 152, 154, 155 and 159) were found to show some homology to previously identified ESTs. An additional ten clones (SEQ ID NO: 161-170) were found to have some degree of homology to known genes. An additional clone, referred to as P703, was found to have five splice variants. The determined DNA sequence for the variants referred to as DE1, DE13 and DE14 are provided in SEQ ID NOS: 171, 175 and 177, respectively, with the corresponding predicted amino acid sequences being provided in SEQ ID NO: 172, 176 and 178, respectively. The DNA sequences for the splice variants referred to as DE2 and DE6 are provided in SEQ ID NOS: 173 and 174, respectively.

mRNA Expression levels for representative clones in tumor tissues (prostate (n=5), breast (n=2), colon and lung) normal tissues (prostate (n=5), colon, kidney, liver, lung (n=2), ovary (n=2), skeletal muscle, skin, stomach, small intestine and brain), and activated and non-activated PBMC was determined by RT-PCT as described above. Expression was examined in one sample of each tissue type unless otherwise indicated.

P9 was found to be highly expressed in normal prostate and prostate tumor compared to all normal tissues tested except for normal colon which showed comparable expression. P20 was found to be highly expressed in normal prostate and prostate tumor, compared to all twelve normal tissues tested. A modest increase in expression of P20 in breast tumor (n=2), colon tumor and lung tumor was seen compared to all normal tissues except lung (1 of 2). Increased expression of P18 was found in normal prostate, prostate tumor and breast tumor compared to other normal tissues except lung and stomach. A modest increase in expression of P5 was observed in normal prostate compared to most other normal tissues. However, some elevated expression was seen in normal lung and PBMC. Elevated expression of P5 was also observed in prostate tumors (2 of 5), breast tumor and one lung tumor sample. For P30, similar expression levels were seen in normal prostate and prostate tumor, compared to six of twelve other normal tissues tested. Increased expression was seen in breast tumors, one lung tumor sample and one colon tumor sample, and also in normal

PBMC. P29 was found to be over-expressed in prostate tumor (5 of 5) and normal prostate (5 of 5) compared to the majority of normal tissues. However, substantial expression of P29 was observed in normal colon and normal lung (2 of 2). P80 was found to be over-expressed in prostate tumor (5 of 5) and normal prostate (5 of 5) compared to all other normal tissues tested, with increased expression also being seen in colon tumor.

Further studies using the above methodology resulted in the isolation of sixteen additional clones. The determined DNA sequences for these clones are provided in SEQ ID NO: 207-222.

EXAMPLE 4

SYNTHESIS OF POLYPEPTIDES

Polypeptides may be synthesized on an Applied Biosystems 430A peptide synthesizer using FMOC chemistry with HPTU (O-Benzotriazole-N,N,N',N'-tetramethyluronium hexafluorophosphate) activation. A Gly-Cys-Gly sequence may be attached to the amino terminus of the peptide to provide a method of conjugation, binding to an immobilized surface, or labeling of the peptide. Cleavage of the peptides from the solid support may be carried out using the following cleavage mixture: trifluoroacetic acid:ethanedithiol:thioanisole:water:phenol (40:1:2:2:3). After cleaving for 2 hours, the peptides may be precipitated in cold methyl-t-butyl-ether. The peptide pellets may then be dissolved in water containing 0.1% trifluoroacetic acid (TFA) and lyophilized prior to purification by C18 reverse phase HPLC. A gradient of 0%-60% acetonitrile (containing 0.1% TFA) in water (containing 0.1% TFA) may be used to elute the peptides. Following lyophilization of the pure fractions, the peptides may be characterized using electrospray or other types of mass spectrometry and by amino acid analysis.

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for the purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention.